



Amino acid sequence and prion strain specific effects on the *in vitro* and *in vivo* convertibility of ovine/murine and bovine/murine prion protein chimeras

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Abstract

Prion diseases are characterised by the conversion of a cellular prion protein (PrP^C) by its misfolded, hence pathogenic, isoform (PrP^{Sc}). The efficiency of this transition depends on the molecular similarities between both interaction partners and on the intrinsic convertibility of PrP^C. Transgenic mice expressing chimeric murine/ovine PrP^C (Tgmushp mice) are susceptible to BSE and/or scrapie prions of bovine or ovine origin while transgenic mice expressing similar murine/bovine PrP^C chimera (Tgmubo mice) are essentially resistant. We have studied this phenomenon by cell-free conversion on procaryotically expressed chimeric PrP^C. Mouse passaged scrapie or BSE PrP^{Sc} was used as a seed and the conversion reaction was carried out under semi-native conditions. The results obtained in this assay were similar to those of our *in vivo* experiments. Since mubo- and mushp-PrP^C differ only at four amino acid positions (S96G, N142S, Y154H and Q185E), single or double point mutations of mushp-PrP^C were examined in the cell-free conversion assay. While the scrapie Me7 prion induced conversion was largely reduced by the N142S and Q185E but not by the S96G and Y154H mutation, the BSE induced conversion was retained in all mutants. Newly formed PrP^{res} exhibited strain specific characteristics, such as the localisation of the proteinase K cleavage site, even in the chimeric PrP^C mutants. We therefore postulate that the efficiency of the conversion of chimeric PrP^C depends on the amino acid sequence as well as on prion strain specific effects.

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1. Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurological disorders which include among others, scrapie in small ruminants, BSE in cattle and Creutzfeldt–Jacob disease in humans. Prion diseases are supposedly caused by the conversion of the cellular prion protein (PrP^C) into an abnormal conformational isoform (PrP^{Sc}) [1,2]. However, the molecular mechanism of this conversion process is still not fully understood. The formation of PrP^{Sc} is associated with an increase in the β -sheet content of the prion protein. This results in a partial resistance to proteases such as proteinase K (PK) and the tendency to form amyloid fibrils. Due to this insolubility, a high-resolution structure of PrP^{Sc} cannot

be analysed to date, whereas the structure of the α -helical and hence more soluble PrP^C has been revealed by nuclear magnetic resonance analysis [3].

Various studies of familial human TSEs have indicated a coherence between the amino acid sequence of the host PrP^C and both the incubation time and the disposition to develop a spongiform encephalopathy [4]. There are about 20 point mutations or other polymorphisms known to be associated with the occurrence of human TSEs [5]. It has also been demonstrated that the substitutions of few or even single amino acids have the ability to alter either the incubation time or actually the probability to develop clinical disease [6].

In sheep nine different alleles/polymorphisms out of the 256 amino acids of the prion protein have been described. Out of these, the polymorphisms at amino acids 136, 154 and 171 have been linked with the susceptibility to classical scrapie and BSE infections and are currently used in the European Union for breeding programs to generate resistant sheep [7,8]. Another

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polymorphism at amino acid 141 is linked to the susceptibility to atypical scrapie [9]. Other than in sheep only one amino acid polymorphism (introduction of an additional six octapeptide sequence) has been revealed in cattle which, however, is not modulating the BSE prion susceptibility [10].

The molecular mechanism by which these mutations in PrP control the incubation times is not as yet understood. The species barrier phenomenon, i.e. the reduced efficiency of prion transmission to a new host species, is primarily believed to result from the inefficient interaction between PrP^{Sc} and PrP^C from the two different species. Moreover, independent from this effect a variety of different prion strains has been found. Some strains propagate only inefficiently in some animal species, while others replicate very well. Prion strains can also be distinguished by distinct incubation times in different host species, by distinct neuropathological lesion profiles in defined mouse lines and, as revealed by western blot analysis, by differences in the PK resistance and in the glycosylation patterns of the abnormal PrP^{Sc} [11,12].

In order to overcome the species barrier for the transmission of human or ruminant derived prions to mice, a number of transgenic mouse models have been developed in which the human, bovine or ovine PrP^C is overexpressed [14,15,17,18]. For example, Tgbov XV mice exhibit significantly shorter incubation times following inoculation with cattle derived BSE prions (220–240 days) than conventional RIII mice (well over 330 days) [14]. Moreover, these incubation times are not further shortened during the secondary passage. Furthermore, by parallel end point titrations it was shown that these mice are approximately 10,000 times more susceptible to a BSE infection than RIII mice and are about ten times more susceptible than cattle [16]. However, in humanized and bovinized transgenic mice this PrP^C replacement strategy only worked well when the endogenous mouse PrP^C expression was ablated [17]. On the other hand, human prions could also be efficiently transmitted into transgenic mice expressing a

chimeric PrP^C in which the core part of murine PrP^C (amino acids 94–188 of hamster PrP) was substituted by human sequences when they coexpressed wild-type murine PrP^C [18]. This phenomenon gave rise to the protein X hypothesis which postulates that murine cofactors obligatorily needed for the human PrP^C conversion would bind with high affinity to murine PrP^C, if coexpressed and thereby block the conversion of the transgene-encoded PrP^C. As human/murine chimeric PrP^C expressing transgenic mice were equally susceptible to a CJD infection, it was concluded that the authentic binding sites for protein X would also be provided by the chimeric protein.

Chimeric mushp-PrP^C overexpressing transgenic mice were almost equally susceptible to ovine and bovine TSE isolates as wild-type ovine PrP^C overexpressing transgenic mice (results described in this paper). However, a different effect was observed in bovine/murine chimeric PrP^C overexpressing transgenic mice which were almost completely resistant to a BSE infection, even in the absence of an endogenous murine PrP^C expression [13,16]. Interestingly, mubo-PrP^C and mushp-PrP^C differ in just four amino acids at residues 96, 142, 154 and 185 (Fig. 1). Therefore we used these models to examine the influence of the primary structure of PrP^C on the protein interaction and the convertibility during the initial steps of the prion cascade. For this analysis we used a cell-free conversion assay in which the conversion reaction can be mimicked by incubating highly purified PrP^C molecules together with PrP^{Sc} seeds in an appropriate conversion buffer [19]. Under these conditions it was shown that PrP^{Sc} itself is able to induce the conversion of PrP^C into a partially proteinase K resistant form which is designated PrP^{res}. This newly formed PrP^{res} can be selectively detected and quantified by an antibody that reacts to an epitope tag which is absent in the original PrP^{Sc} seed [20,21]. As the results obtained using the cell-free assay were generally in line with *in vivo* data, this method offers the possibility to simulate – in a first approach – PrP^C conversion

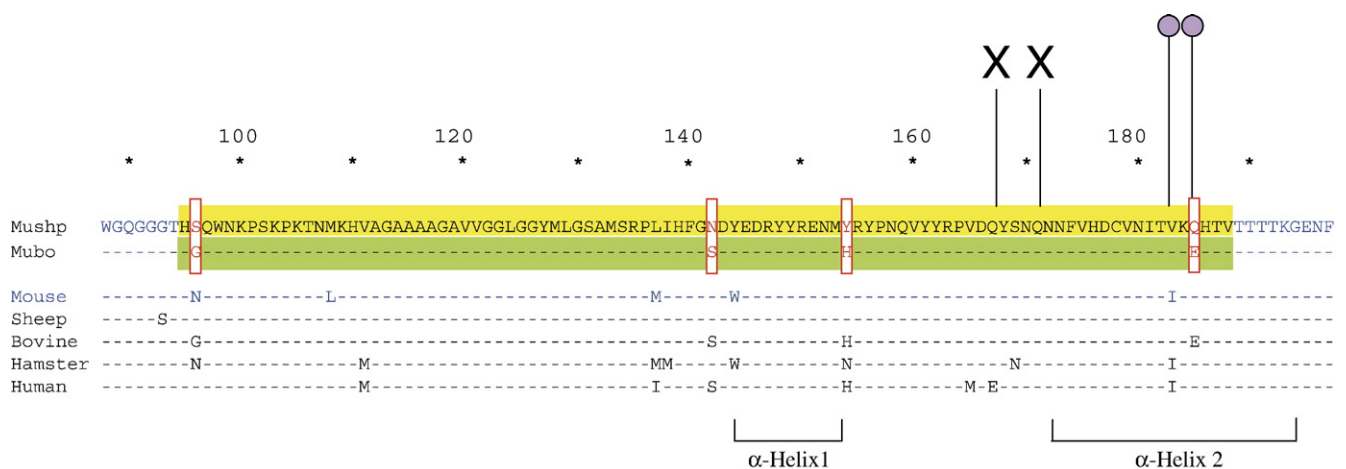


Fig. 1. Amino acid sequence alignment of mouse, hamster, human bovine and sheep prion protein (residues 90–232) as well as of bovine/mouse and ovine/mouse chimera. Residues demarked 'X' specify the proposed protein X binding sites, while residues 183 and 185 are supposed to determine the primary PrP interaction sites which are responsible for intra- and inter-species binding and barrier effects in bovine PrP. Chimeric PrP^Cs are composed of murine sequences (blue) into which sheep sequences (mushp, yellow) or bovine sequences (mubo, green) were introduced between amino acids 94 and 188. Mubo and mushp differ in four amino acids (red), whose effects on the convertibility were examined.

reactions involved in prion diseases without performing more time, work and life consuming transgenic mouse challenge experiments.

2. Materials and methods

2.1. Generation of chimeric transgenic mice

The generation and characterisation of murine/bovine PrP transgenic mice (Tgmubo XIII) has been described earlier [14].

Murine/ovine PrP transgenic mice (Tgmushp XIX) were prepared accordingly. Briefly, the open reading frame sequence coding for the core region of the ovine PrP from aa 94 to 188 of murine PrP (98 to 192 of ovine PrP) with the amino acids alanine (A), arginine (R), and glutamine (Q) at positions 136, 154 and 171 (PrP^{ARQ} allele) was PCR amplified. This PCR step introduced a *KpnI* digestion site and at the same time a point mutation from serine (ovine sequence) to threonine (murine sequence) at codon 98 of the ovine PrP sequence (codon 94 of the murine PrP) by PCR-mutagenesis using the primers ovPrPHG2.seq 5' GGG GTC AAG GTG GTA CCC ACA GTC 3' and ovPrPHG3.rev 5' GGT GGT GGT GAC CGT GTG TTG C 3' leaving the *BstEII* digestion site at codon 189 (codon 193 of ovine PrP) intact. The fragment was then cloned into the vector muboPrPHG coding for the murine/bovine chimeric PrP [14] that had been digested with *KpnI* and *BstEII*. The resulting transgene construct mushpPrPHG was microinjected into oocytes of PrP^{0/0} mice.

Five transgenic mouse lines were identified that expressed the murine/ovine chimeric PrP in their brains at a level that was at least as high as in the ovine brain, out of which the mouse line Tgmushp XIX displayed with 8–16 fold the highest overexpression. Therefore this mouse line was selected for comparative mouse bioassays.

2.2. Transgenic mouse challenge studies

Challenge studies were performed to compare the susceptibility of Tgmubo and Tgmushp mice with that of the respective mouse lines expressing full-length ovine and bovine PrP. One line of each transgene construct with the highest brain expression level was selected for comparative transmission studies. These were the lines Tgbov XV (16 fold overexpression), Tgmubo XIII (16 fold overexpression), Tgshp XI (4–8 fold overexpression) and Tgmushp XIX (8–16 fold overexpression). All challenged mice were on a PrP-ablated background. Ten or 15 mice (depending on the availability of inoculum and on the expected survival times of the mice) were inoculated intracerebrally (i.c.) with 20 µl plus 100 µl intraperitoneally (i.p.) of a 10% brain homogenate of the following BSE and scrapie isolates: BSE BBP12/92 which had already been used for a parallel BSE titration experiment in Tgbov XV mice, a German field scrapie isolate designated 'Potsdam' derived from an animal carrying the PrP^{ARQ/ARQ} allele, and the mouse passaged scrapie strains Me7 (passaged in C57Bl mice), 22A (passaged in C57Bl mice), and Chandler (passaged in CD1 mice).

Mice were supervised at least twice weekly for the onset of clinical symptoms. Animals showing signs of disease were sacrificed and the mouse brains were tested for the accumulation of PrP^{Sc} by western blot or immunohistochemical methods.

2.3. Detection of PrP^{Sc} accumulation in the brains of challenged transgenic mice

2.3.1. Preparation of scrapie-associated fibrils (SAF) and immunoblot

Two hundred microliters of a 10% mouse brain homogenate were incubated with 50 µg/ml proteinase K (PK; Roche, Mannheim, Germany) for 60 min at 37 °C to completely digest all PrP^C. The reaction was stopped by the addition of 10 mM PMSF (phenylmethylsulfonylfluoride) and incubated at 95 °C for 5 min. After adding sarcosine to a final concentration of 10% and Tris–HCl (pH 7.4) to 10 mM, the samples were incubated at room temperature for 15 min. The samples were then carefully loaded over 250 µl of a 10 mM Tris–HCl solution (pH 7.4) containing 10% sucrose and centrifuged at 540 000 g for 45 min. The pellets were resuspended in 40 µl gel loading buffer containing 2% SDS and incubated at 95 °C for 5 min.

2.3.2. Immunohistochemical examination

Mouse brain sections were pre-treated (incubation of the slides in formic acid for 15 min, incubation with 4 µg/ml Proteinase K at 37 °C for 15 min and hydrated autoclaving for 15 min) in order to improve the accessibility of the antibody epitopes. Slides were then incubated with mab L42 [22] at an appropriate concentration. A biotinylated goat-anti-mouse conjugate (Vector, Burlingame, CA, USA) visualized the binding of the primary antibody. For signal detection, an avidin–biotin–complex system (Vectastain ABC Elite Kit) in combination with the VIP substrate (Vector, Burlingame) was applied.

2.4. Mutation of Prnp

Amino acids at residues 96, 142, 154 and 185 of mushp-PrP^C were substituted by those of mubo-PrP^C. The mutations were carried out by a two step PCR using first internal primers for the particular substitution and external primers for the amplification of the mutant template in a second step. The mutant mushp-PrP^C was appropriately named depending on the particular substitution.

Using the primers which are listed below and Prnp^{mushp} as a template, point mutations were introduced to alter residues 96 (mushp-PrP^C S96G), 142 (mushp-PrP^C N142S), 154 (mushp-PrP^C Y154H) and 185 (mushp-PrP^C Q185E).

Mutation at residue 96 was carried out by the use of 5'-GGT ACC CAC GGT CAG TGG AAC-3' as N-terminal internal primer and 5'-GTT CCA CTG ACC GTG GGT ACC-3' as C-terminal internal primer. Mutation at residue 142 was carried out using 5'-CAT TTT GGC AGT GAC TAT GAG-3' as N-terminal internal and 5'-CTC ATA GTC ACT GCC AAA ATG-3' as C-terminal internal primer. Mutation at residue 154 was carried out using 5'-GAA AAC ATG CAC CGT TAC CCC-3' as N-terminal internal primer and 5'-GGG GTA ACG GTG CAT GTT TTC-3' as C-terminal internal primer. Finally mutation at residue 185 was carried out using 5'-ACA GTC AAG GAA CAC ACA GTC-3' as N-terminal internal primer and 5'-GAC TGT GTG TTC CTT GAC TGT-3' as C-terminal internal primer.

5'-CG GGA TCC AAA AAG CGG CCA-3' and 5'-GG AAG CTT GGA TCT TCT CCC GTC G-3' were used as external primers.

Successive mutations were accomplished by the use of Prnp^{mushp} S96G as a template and the primers for substitution of residue 142 (mushp-PrP^C S96G/N142S) and mushp-PrP^C S96G/N142S as a template and the primers for mutation at residue 154 respectively mushp-PrP^C S96G/N142S/Y154H).

2.5. Expression and purification of recombinant PrP^C

The gene coding for either mubo-PrP^C or mushp-PrP^C was amplified from the ORF 23–231 by PCR using the primers 5'-CG GGA TCC AAA AAG CGG CCA-3' and 5'-GG AAG CTT GGA TCT TCT CCC GTC G-3'. Using the *Bam* HI and *Hind*III cleavage sites, the amplified gene was cloned into the *E. coli* vector pQE 40 (Qiagen) which harbours a N-terminal histidine (his)-tag. Expression was carried out in Mi 5-cells (Qiagen) which were cultivated at 37 °C in 500 ml LB medium to an optical density of 0.6 at 600 nm. After the addition of Isopropyl-β-D-thiogalactopyranoside (to a final concentration of 1 mM) the cultures were grown for another 4.5 h and the bacteria harvested by centrifugation at 3600g and 4 °C for 10 min. Purification was carried out according to the manufacturer's instruction under denaturing conditions (8 M Urea). Refolding was accomplished by dialysis in 20 mM HEPES (Roth) containing 0.2% 5 M EDTA and 0.1% N-lauroylsarcosine sodium salt (Sigma Aldrich).

2.6. Preparation of PrP^{Sc}

PrP^{Sc} was purified from terminally scrapie diseased mouse brains (C57Bl/6) (mouse scrapie Me7 as well as mouse passaged BSE). The preparation was performed according to the method described by Caughey et al. [19] with some modifications. Briefly, 1 g mouse brain samples were homogenised on ice in 15 ml TEND buffer (10 mM Tris, pH 8, 3, 1 mM EDTA, 130 mM NaCl) supplemented with 10% N-lauroylsarcosine sodium salt as well as protease inhibitors (1 mM DTT, 0.1 mM Pefabloc, 0.5 µg/ml Leupeptin, 1 µg/ml Aprotinin, 0.7 µg/ml Pepstatin) and centrifuged for 30 min at 23 000 rpm (4 °C) in a TLA 100.4 rotor (Beckman). The supernatant was collected and centrifuged for 2.5 h at 58 000 rpm (4 °C). The resulting pellet was resuspended in 15 ml TEND buffer containing 10% NaCl and 1% Sulfobetain SB-14 and centrifuged

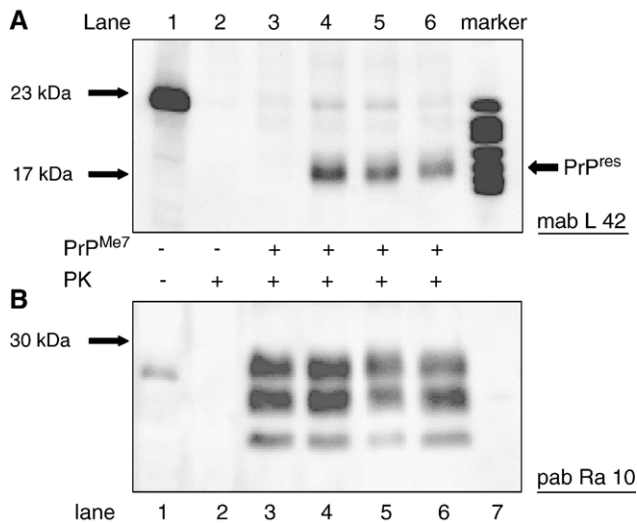


Fig. 2. (A) Immunoblot of a cell-free conversion reaction using ARQ. The newly formed PrP^{res} fragment is detected by mab L42, if recombinant PrP^{C} (lane 1) changed its conformation into a partially PK resistant form, which is characterised by a molecular mass of about 17 kDa after PK treatment (lanes 4–6). As mab L42 does not detect mouse PrP^{Sc} , the blotting membranes were washed with glycine buffer pH 2.0 to wash off mab L42 prior to an incubation with the polyclonal antibody Ra10 (B) which detects the murine Me7- PrP^{Sc} seed (lanes 3–6).

at 68 000 rpm for 1.5 h at 20 °C. The supernatant was decanted and the pellet was resuspended in 8.8 ml TMS buffer which was supplemented with 10% NaCl and 0.5% SB-14. After homogenisation the suspension was carefully loaded onto a sucrose cushion (1.0 M sucrose, 0.1 M NaCl, 0.5% SB-14) and centrifuged at 68 000 rpm for 1.5 h at 20 °C. The remaining pellet was resuspended in 200 μl PBS containing 0.5% SB-14, homogenised, sonified and finally stored at –20 °C.

2.7. PrP cell-free conversion assay

The conversion reaction was carried out using equimolar amounts of PrP^{C} and PrP^{Sc} as determined by immunoblotting using a recombinant PrP^{C} standard. Generally, the amounts of protein used were between 500 and 800 μg . After an incubation for 3 days at 37 °C in a defined semi-native buffer (50 mM citrate pH 6.0, 200 mM KCl, 5 mM MgCl_2 , 1.25% N-lauroylsarcosine) the samples were treated with proteinase K (PK) diluted in TN buffer (0.15 M NaCl, 0.05 M Tris–HCl pH 7.4) to a concentration of 30 $\mu\text{g}/\text{ml}$. After 1 h at 37 °C the reaction was stopped with 10 mM phenyl methanesulfonyl fluoride (PMSF). A carrier protein (20 μg thyroglobuline) was added to the samples prior to an incubation with 200 μl methanol at –20 °C for at least 1 h. Precipitated proteins were pelleted by centrifugation at 13 000 rpm for 15 min (20 °C) in a table top centrifuge. Each experiment included control samples which did not contain any PrP^{Sc} (Fig. 2, lanes 1, 2), which were not digested with PK (lane 1) or which were not incubated for 3 days at 37 °C but instead immediately stored at –20 °C (lane 3).

Table 1

Average incubation times in days of transgenic mice that had accumulated PrP^{Sc} in their brains (as detected by immunoblot and/or immunohistochemistry) after i.c. and i.p. inoculation of BSE and scrapie

Mouse line	Inoculum	Sheep scrapie ‘Potsdam’	BSE BBP12/92	ME7	22A	Chandler
Tgbov XV		195 8/8 SEM=5.74	230 7/7 SEM=7.48	>377 0/4	>769 0/2	>470 0/6
Tgmubo XIII		555 4/9 SEM=47.48	599 6/10 SEM=77.64	>610 0/5	565 6/7 SEM=28.03	749 2/9 SEM=0
Tgshp XI		202 4/4 SEM=11.96	305 5/5 SEM=29.58	209 6/6 SEM=17.41	282 5/8 SEM=20.93	224 5/5 SEM=20.14
Tgmushp XIX		259 7/9 SEM=12.42	382 9/9 SEM=18.73	182 4/4 SEM=1.6	358 5/5 SEM=16.71	152 15/15 SEM=7.89

SEM=standard error of the mean.

All animals were euthanized after the onset of clinical symptoms or after 800 days at the end of the experiment.

2.8. SDS-PAGE and immunoblotting

Following centrifugation the pellets were resuspended in a loading buffer (1% SDS, 25 mM Tris/HCl pH 7.4, 0.5% mercaptoethanol, 0.001% bromophenolblue) and incubated at 95 °C for 5 min. 15 μl of each sample was separated in a 16% acrylamide gel. Proteins were then transferred onto PVDF membranes by semi-dry electroblotting. The membranes were incubated with blocking buffer (PBS, 0.1% Tween, 5% skimmed milk powder) at room temperature for 30 min, followed by an incubation for 1 h with the monoclonal antibody mab L42 [22] that specifically binds to ruminant PrP but not to murine PrP in order to detect the newly formed PrP^{res} . Afterwards, the membranes were washed three times for 10 min in PBS, 0.1% Tween. The proteins were visualized by using an alkaline phosphatase conjugated goat anti-mouse secondary antibody and the chemiluminescence detection substrate CDP-Star (Tropix).

After treating the membranes with power strip buffer (0.2 M glycine, 1% SDS, pH 2) two times for 10 min PrP^{Sc} was detected by using a polyclonal antibody pab Ra10 [23,24] that does not detect ruminant PrP , but specifically binds to murine PrP , and visualized by goat anti-rabbit AP and CDP-Star.

2.9. Quantification and determination of molecular mass

Chemiluminescence signals were detected using the BioRad VersaDoc™ photo imaging system and analysed by the Quantity One quantification software (BioRad). The conversion rate was defined as the average rate of five independent reactions. Efficiencies were calculated as the percentages of PrP^{C} converted into newly formed PrP^{res} .

Determination of molecular masses was carried out using a custom-designed protein ladder, which ranged between 16 and 23 kDa in steps of 1 kDa (FLI marker). Each of the proteins harboured an amino-terminal his-tag and could therefore be detected by an anti-histidine antibody (RGS-His., Qiagen).

3. Results

3.1. Generation and challenge of transgenic mice

Chimeric murine/ovine (Tgmushp XI) and murine/bovine PrP^{C} (Tgmubo XIII) overexpressing transgenic mice were generated in which residues 94–188 represented ruminant PrP^{C} . Transgenic lines were selected in which the transgene expression was about 8–16 fold higher than the PrP^{C} expression in nontransgenic mice. All transgenic mice used in these challenge experiments were on a wild-type PrP^{C} ablated genetic background. These challenge experiments showed that chimeric Tgmushp XIX mice are almost as susceptible to cattle derived BSE, to sheep derived scrapie as well as to mouse passaged scrapie 22A as their non-chimeric counterpart, Tgshp XI. Meanwhile, their susceptibility to the mouse passaged scrapie strains Me7 and Chandler was

significantly higher than that of Tgshp XI mice. On the other hand, Tgmubo XIII mice displayed long incubation times of over 550 days after being challenged with BSE and field scrapie as well as the mouse passaged scrapie strain 22A. These mice proved to be essentially resistant to an infection with the mouse passaged scrapie strains ME7, where no mouse developed disease at all, and Chandler, where two out of seven mice developed disease after 749 days. Comparing the two transgenic mouse lines expressing the different

chimeric PrP constructs, Tgmushp XIX mice show distinctly shorter incubation times than Tgmubo XIII mice after challenge with all five inocula. The most interesting difference, however, was seen after challenge with the scrapie strains ME7 and Chandler, that induced very short incubation times of 182 and 152 days in Tgmushp XIX mice and that hardly induced disease at all in Tgmubo XIII mice although these two mouse lines only differ in four amino acids of the expressed prion protein (Table 1).

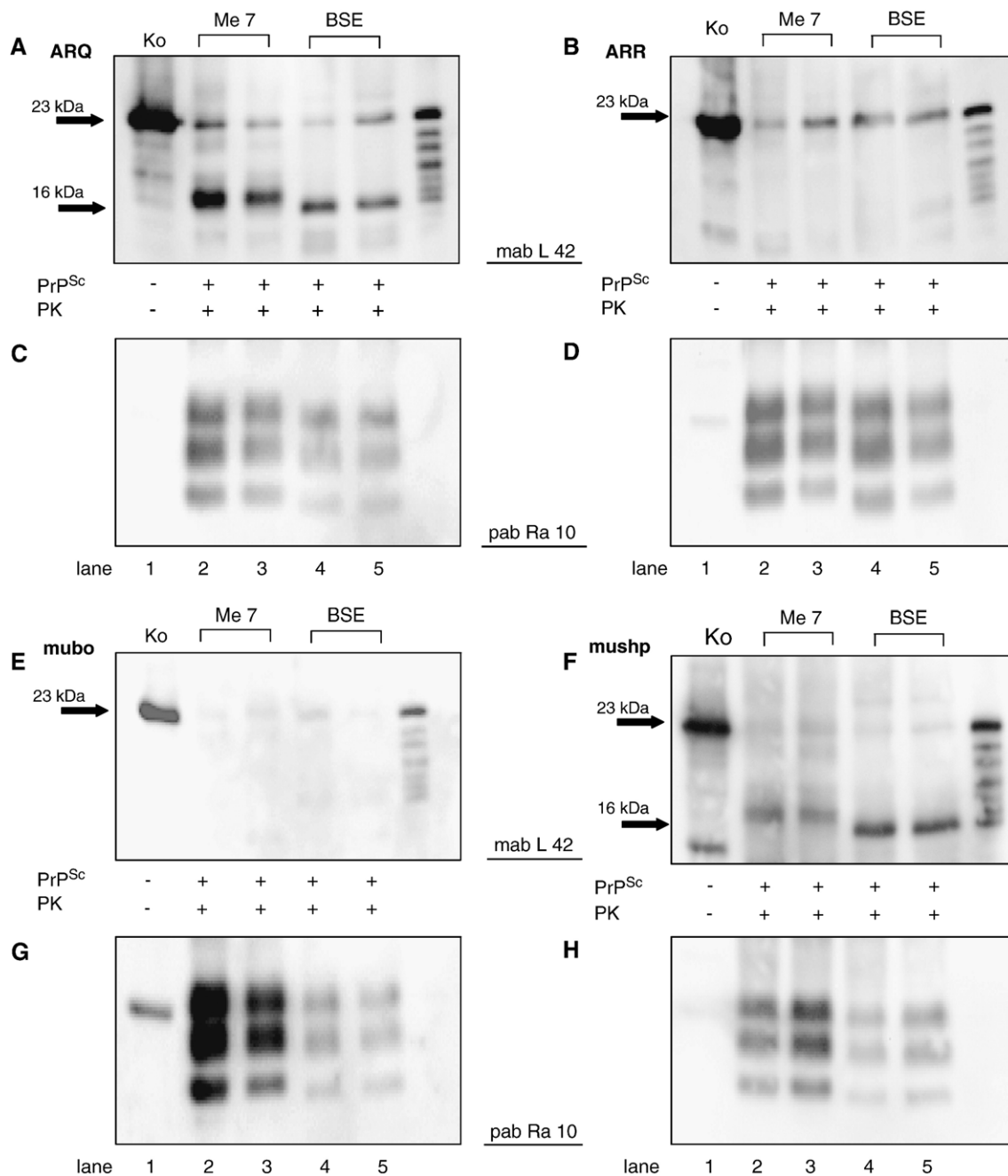


Fig. 3. Immunoblots of recombinant sheep prion protein allelic variants PrP^{ARQ} (A) and PrP^{ARR} (B) after incubation with Me7-PrP^{Sc} (C, D: lanes 2, 3) and BSE-PrP^{Sc} (C, D: lanes 4, 5). The difference of about 1 kDa in the molecular mass of the PrP^{Sc} seed is transmitted to the particular PrP^{Sc}. Immunoblots of recombinant chimeric mubo-PrP^C and mushp-PrP^C (E, F) after incubation with Me7-PrP^{Sc} (G, H: lanes 2, 3) and BSE-PrP^{Sc} (G, H: lanes 4, 5). PrP^{Sc} can be detected in the case of mushp-PrP^C (F: lanes 2–5) whereas there is no comparable fragment detected in the case of mubo-PrP^C (E).

3.2. Mouse Me7-PrP^{Sc} induced conversion of chimeric PrP^C to PrP^{res}

The intrinsic convertibility of chimeric mushp-PrP^C and mushp-PrP^C was probed in a cell-free conversion assay under semi-native (i.e. non-denaturing) conditions, where purified mouse Me7-PrP^{Sc} was used as a seed and structurally renatured

and affinity purified procaryotic PrP^C was used as a template. PrP^C from sheep carrying the PrP^{ARQ} (easily convertible into the pathogenic isoform) or the PrP^{ARR} (not convertible under the applied conditions) allele in homozygosity were used as positive and negative controls for the conversion assay (Figs. 2, 3A–D). After an incubation of mushp-PrP^C as a template and mouse Me7-PrP^{Sc} as a seed for 3 days at 37 °C, a PrP^{res} fragment of

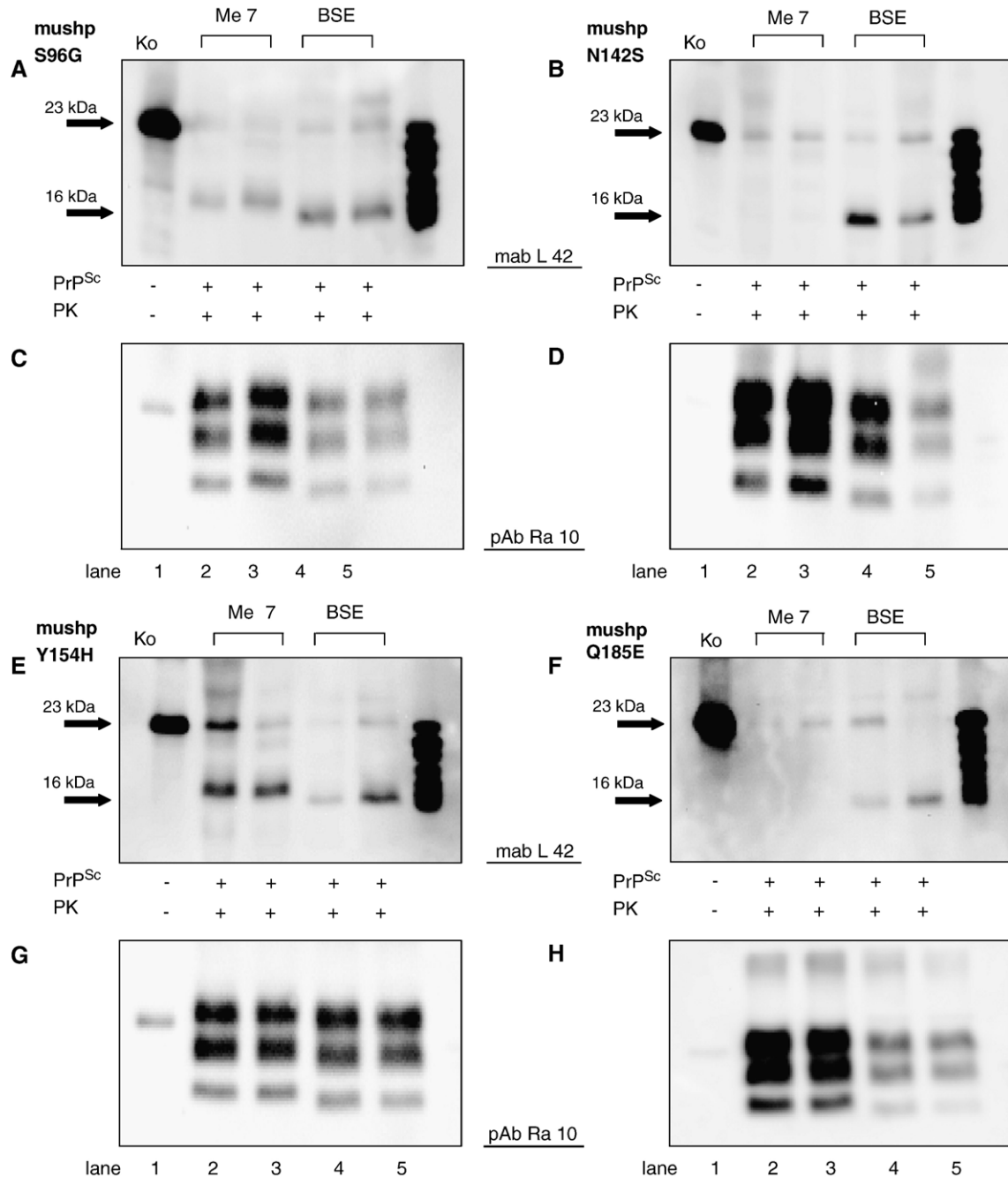


Fig. 4. Immunoblots of recombinant chimeric mushp-PrP^CS96G (A), mushp-PrP^CN142S (B), mushp-PrP^CY154H (E) and mushp-PrP^CQ185E (F) after incubation with Me7-PrP^{Sc} (lanes 2, 3) or BSE-PrP^{Sc} (lanes 4, 5). PK resistant PrP is visualized by using mab L42. Immunoblot lanes on panels C, D, G and H depict the corresponding PrP^{Sc} used as seed, which was visualized by a specific polyclonal antibody Ra10. Even though the BSE-PrP^{Sc} signal on the immunoblot is not as strong as the one of Me7-PrP^{Sc} (C, D), BSE-PrP^{Sc} induces a conversion of mushp-PrP^CN142S and mushp-PrP^CQ185E into a partially PK resistant fragment (B, F: lanes 4, 5), while Me7-PrP^{Sc} was not able to induce a conversion in mushp-PrP^CN142S or mushp-PrP^CQ185E into PrP^{res} (B, F: lanes 2, 3). Mushp-PrP^CS96G and mushp-PrP^CY154H were converted by both seeds, Me7-PrP^{Sc} as well as BSE-PrP^{Sc} (A, E: lanes 2–5). The slight difference in the molecular mass between Me7-PrP^{Sc} and BSE-PrP^{Sc} is also transferred to the PrP^{res} of the mutant chimeric mushp-PrP^C.

about 17 kDa was detected in western blot analysis using mab L42. In contrast, no PrP^{res} was detectable after the same treatment when mubo-PrP^C was used as a template (Figs. 3E–H). These cell-free conversion results were in accordance with the *in vivo* findings described above.

3.3. Conversion of mushp-PrP^C mutants

Mushp- and mubo-PrP^C differ in only four amino acids, at positions 96, 142, 154 and 185 (Fig. 1). In order to find out which of these four polymorphisms were responsible for the resistance of mubo-PrP^C, we mutated each of these amino acids in mushp-PrP^C, either alone or in different combinations. Residue 96 is positioned in the flexible N-terminal region of PrP^C, which is not well structured. Serine was substituted by glycine in mushp-PrP^CS96G. Residue 142 is located in the vicinity of the first α -helix in the loop close to the third helix. Asparagine was replaced by serine in mushp-PrP^CN142S. Mushp-PrP^CY154H was created by the substitution of tyrosine by histidine. This residue is localised at the end of the first α -helix and the amino acid side chain points to the outside of the PrP^C molecule. Glutamine was replaced by glutamic acid in mushp-PrP^CQ185E at residue 185 which is located in the second half of α -helix two. In the three-dimensional structure of PrP, glutamine as well as glutamic acid point towards the first structured parts of PrP^C.

The convertibility of mutant mushp-PrP^C into its PrP^{res} counterpart was not affected when serine was changed to glycine at residue 96 (mushp-PrP^CS96G) (Fig. 4A) or when tyrosine was changed to histidine at residue 154 (mushp-PrP^CY154H) (Fig. 4E). However, when residue 142 was changed from asparagine to serine (mushp-PrP^CN142S) (Fig. 4B) or when residue 185 was changed from glutamine to glutamic acid (mushp-PrP^CQ185E) (Fig. 4F), the formation of a PrP^{res} fragment could not be detected.

In the next step, we inserted combinations of several mutations in one protein. After the substitution from serine to glycine at residue 96 and asparagine to serine at residue 142 (mushp-PrP^C 96–142) there was no conversion detectable (Fig. 6B). Moreover, when tyrosine was additionally changed to histidine at residue 154 (mushp-PrP^C 96–142–154), PrP^C was also inconvertible (Fig. 6B).

Table 2

Overview of the chimeric PrP^Cs which were examined for their convertibility using either Me7-PrP^{Sc} or BSE-PrP^{Sc} as seeds

Identification of PrP ^C	Convertibility (Me7-PrP ^{Sc})	Convertibility (BSE-PrP ^{Sc})
Mubo	–	–
Mushp	+	+
Mushp S96G	+	+
Mushp N142S	–	+
Mushp Y154H	+	+
Mushp Q185E	–	+
Mushp 96–142	–	+
Mushp 96–142–154	–	+

Mubo-PrP^C was the only construct which was not converted by any of the PrP^{Sc} seeds.

These results indicate that point mutations at amino acid residues 142 and 185, separately or in combinations, have a negative influence on the convertibility of chimeric mushp-PrP^C to PrP^{res} when Me7-PrP^{Sc} is used as a seed (Fig. 4B, D; F, H).

3.4. Mouse derived BSE-PrP^{Sc} induced conversion of chimeric PrP^C to PrP^{res}

Results obtained with mouse passaged BSE-PrP^{Sc} seeds and mushp-PrP^C or mubo-PrP^C were identical to those obtained with ME7-PrP^{Sc}, i.e. mushp-PrP^C was convertible, while mubo-PrP^C was not (Fig. 3E, F).

3.5. Mouse derived BSE-PrP^{Sc} conversion of chimeric PrP^C mutants

Unlike mouse scrapie Me7-PrP^{Sc} mouse passaged BSE induced a conversion of all mutant chimeric PrP^C constructs (Fig. 4). The strain specific difference of approximately 1 kDa in molecular mass after PK digestion (Fig. 5) was maintained after the *in vitro* conversion. The newly formed PrP^{res} was approximately 1 kDa lower in molecular mass when BSE-PrP^{Sc} was used as a seed than the PK digested PrP^{res} that was newly formed after incubation with Me7-PrP^{Sc} as a seed (Figs. 3, 4).

The results of the convertibility assays of all PrP constructs with Me7-PrP^{Sc} and BSE-PrP^{Sc} are summarized in Table 2.

3.6. Quantitative amounts of PrP^{res}

The relative ratio of converted prion protein was up to seven percent of the amount of PrP^C that was initially set into the reaction (Fig. 6A). Mushp-PrP^C showed almost equal conversion rates after incubation with Me7-PrP^{Sc} or BSE-PrP^{Sc}. Mushp-PrP^CS96G as well as mushp-PrP^CS154H were more efficiently converted by BSE-PrP^{Sc} than by Me7-PrP^{Sc} as the ratio of BSE-PrP^{Sc} induced PrP^{res} was about 1.5 times higher than that of Me7-PrP^{Sc} induced PrP^{res}. This was even more obvious for the mutants mushp-PrP^CN142S, mushp-PrP^CQ185E, mushp-PrP^C96–142 and mushp-PrP^C96–142–154, since Me7-PrP^{Sc} did not provoke any conversion of these mutants, while the conversion rate was approximately

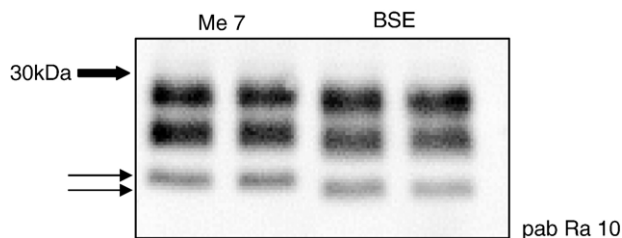


Fig. 5. Immunoblot of Me7-PrP^{Sc} and BSE-PrP^{Sc} after treatment with proteinase K. Due to a difference in the PK cleavage site, which is located further C-terminally in BSE-PrP^{Sc} than it is in Me7-PrP^{Sc} a slight shift in the molecular mass of about 1 kDa can be detected (small arrows) between Me7-PrP^{Sc} and BSE-PrP^{Sc}.

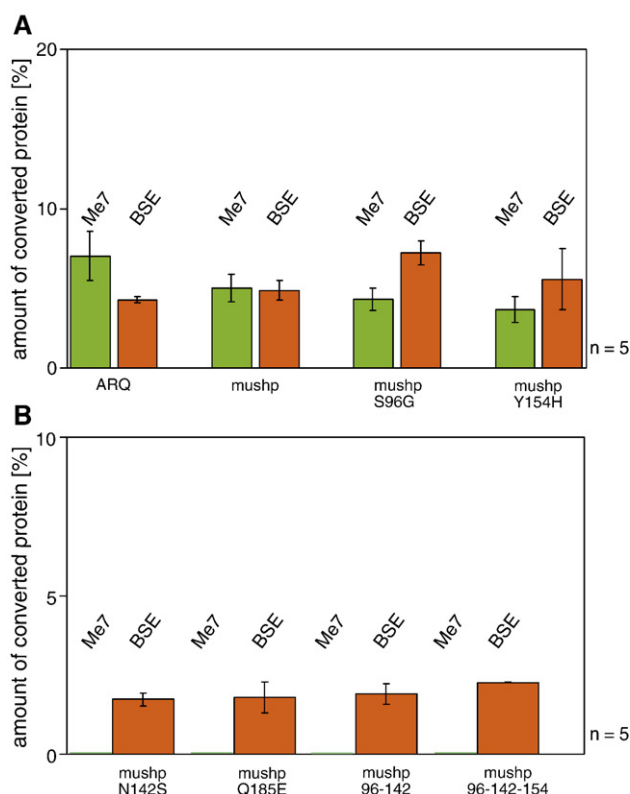


Fig. 6. Quantitative amounts of newly formed PrP^{res} in percent as compared to the total amount of PrP^C which was used in the conversion assay. Concerning the mutant chimeric mushp-PrP^CS96G/mushp-PrP^CY154H the effectivity of conversion is higher if BSE-PrP^{Sc} is used as seed than if Me7-PrP^{Sc} is used. In contrast, the positive control PrP^{ARQ} shows higher amounts of newly formed PrP^{res} if the incubation is carried out with Me7-PrP^{Sc} instead of BSE-PrP^{Sc} (A). Even though Me7-PrP^{Sc} is not able to induce a conversion of the mutant chimeric mushp-PrP^Cs, the quantitative amount of newly formed PrP^{res} which can be detected after an incubation with BSE-PrP^{Sc} ranged at least up to 2% compared to the volume of undigested PrP^C (B).

2% of the total PrP when the reaction was carried out using BSE-PrP^{Sc} as a seed (Fig. 6B).

4. Discussion

We have generated transgenic mice overexpressing either wild-type bovine (Tgbov XV) or ovine (Tgshp XI) PrP^C or murine/bovine (Tgmubo XIII) or murine/ovine (Tgmushp XIX) PrP^C chimera. These animals were challenged with either murine or ovine scrapie or bovine BSE prions. Although mubo-PrP^C and mushp-PrP^C differ in only four amino acids (Fig. 1), Tgmushp XIX mice were highly susceptible, whereas Tgmubo XIII mice were almost resistant to an infection with mouse scrapie ME7 and Chandler. They also displayed a low susceptibility (incubation times of at least 560 days) to bovine BSE, sheep scrapie, and the scrapie strain 22A (Table 1). We therefore postulate the occurrence of strain-specific interactions of PrP^{Sc} with certain amino acid positions of PrP, since the scrapie strains ME7 and Chandler induced very short incubation times of 182 and 152 days in Tgmushp XIX mice while Tgmubo XIII mice hardly developed the disease at all.

The resistance of Tgmubo XIII mice is highly unlikely to be caused by a mismatch at the postulated protein X binding sites at residues 183 and 185 of PrP^C since the highly susceptible Tgbov XV mice encode a protein in which these amino acids are identical to that of Tgmubo XIII mice. Since all mouse lines used in this study showed an expression rate of 8–16 fold, this difference must be caused by the differing four amino acids at positions 96, 142, 154, and 185 between Tgbov XV and Tgmubo XIII PrP^C.

Similar effects were also seen, when a cell-free PrP conversion system was employed. Although this system does not fully reflect the *in vitro* situation, since a procaryotic PrP^C is used which is not posttranslationally modified in the same way as eucaryotically expressed PrP, the applied system is considered appropriate to study the general effects of point mutations on the convertibility of the protein. However, a distinct quantification of the conversion efficiency is difficult within this system. Further studies using more authentic systems such as PMCA are anticipated for the future.

Me7-PrP^{Sc} as well as BSE-PrP^{Sc} were able to induce a conversion of mushp-PrP^C to PrP^{res} in the cell-free assay. In contrast, it was not possible to detect any PrP^{res} when the conversion experiment was carried out with mubo-PrP^C as a template. Thus the difference of the four amino acids at positions 96, 142, 154 and 185 appears to be also responsible for the inconvertibility of mubo-PrP^C *in vitro*. The influence of the amino acid sequence on the convertibility and therefore on the development of disease has already been shown in numerous studies [26,27]. Scrapie susceptibility of sheep is known to be linked to certain PrP genotypes [28]. Especially polymorphisms at codons 136, 154 and 171 are known to be associated with susceptibility and disease phenotype [7]. These findings have been successfully reproduced in *in vitro* systems, either in cell culture or in cell-free conversion assays [21,29]. Since prion proteins of different species only show minor structural modifications [30], it can be assumed that mubo-PrP^C and mushp-PrP^C also do not display major differences in their three dimensional structure. Even though single or few amino acid substitutions do not necessarily change the structural properties or the overall stability of PrP^C [31], they may have an impact on the protein interactions prior to and/or during the conversion process as well as on the binding affinity to yet unknown cofactors. Moreover, the amino acid sequence determines the intrinsic stability of PrP^C and therefore its propensity to conversion and the formation of amyloid fibrils. Such effects have been proposed regarding the allele specific polymorphisms particularly within ovine PrP^C in sheep [32].

As the *in vitro* results were very well in accordance with the *in vivo* findings we tried to elucidate the molecular mechanisms of the conversion reaction by introducing relevant mutations (singular or in combination) at positions 96, 142, 154, or 185 of mushp-PrP^C. These mutants were co-incubated in a cell-free conversion assay using Me7-PrP^{Sc} as a seed. These studies revealed that the mutations at residues 96 and 154 had no effect (Fig. 4A, E), while altering positions 142 and 185 rendered mushp-PrP^C inconvertible by Me7-PrP^{Sc} seeds (Fig. 4B, F).

In mushp-PrP^CS96G serine (uncharged polar side chain) is replaced by glycine (non-polar with a very short side chain). This residue is located in the flexible, structurally not well-defined part of PrP^C. This is probably the reason why this substitution did not have a negative effect on the convertibility of PrP^C in our experiments. However, it has already been reported that the N-terminus of PrP^C does play a role in the prion conversion, as the deletion of amino acids up to residue 124 of hamster PrP resulted in a reduced *in vivo* susceptibility and a lower *in vitro* efficiency of PrP^{res} formation [33].

The Y154H substitution which also had no effect on the cell-free conversion efficiency of mushp-PrP^C in our study using Me7-PrP^{Sc} is located just after the first α -helix. However, it can be assumed that the dipolar character of the α -helix is stronger supported by the presence of tyrosine than it is by histidine, because tyrosine has a polar side chain (phenolic hydroxy-group), whereas histidine carries an uncharged or positive polar side chain.

In contrast, the two mutations N142S and Q185E rendered mushp-PrP^C inconvertible using Me7-PrP^{Sc} (Fig. 4B, F). Asparagine and glutamine at residues 142 (mushp-PrP^CN142S) and 185 (mushp-PrP^CQ185E) are two amino acids which are both known to stabilise secondary structure elements via H-bonds. Residue 142 is located close to the first α -helix residue, whereas 185 lies within the second α -helix. In mushp-PrP^C-N142S asparagine is substituted by serine. Both amino acids exhibit an uncharged polar side chain but serine is noticeably smaller than asparagine. Furthermore, serine misses the terminal carboxamide group which enables asparagine to form H-bonds, which again enhance the intrinsic stability of the molecule. Thus, the exchange might result in a lower stability of mushp-PrP^CN142S and therefore lead to negative results in cell free conversion experiments, as unstable proteins are more easily degraded by endogenous proteases [32]. On the other hand in mushp-PrP^CQ185E glutamine carrying an uncharged polar side chain was substituted by glutamic acid with a charged polar side chain. Like in mushp-PrP^CN142S the replacement of a stabilising amino acid at residue 185 hampered the conversion of this mutated protein into its PrP^{res} counterpart. This is due to the terminally located carboxamide group present in glutamine, since H-bonds can be built both from the oxygen and from the amino group.

In addition to these single point mutations we gradually mutated mushp-PrP^C to mubo-PrP^C by consecutively introducing the amino acids substitutions S96G, N142S, Y154H and Q185E. However, as soon as the S96G substitution was combined with the N142S mutation, mushp-PrP^C lost its convertibility and did not regain it by the additional replacement of Y154H.

It has been reported that the A136V and Q171R mutations in the PrP^{ARQ}, PrP^{ARR} and PrP^{VRQ} alleles which modulate the scrapie resistance of sheep, seem to alter the structural stability of ovine PrP^C by preventing the formation of stabilising H-bonds at the surface of the protein [34]. In accordance with this model we could show that PrP^{ARQ} but not PrP^{ARR} is convertible in the in the cell free conversion assay, independent of whether BSE or Me7 prions are used [21].

All mushp-PrP^C mutants were eventually incubated with mouse passaged BSE-PrP^{Sc}. Surprisingly, the detection of a PrP^{res} fragment was possible in every mutant but not in mubo-PrP^C (Figs. 3, 4). Although Me7-PrP^{Sc} and BSE-PrP^{Sc} harbour the same amino acid sequence (both derived from passages in C57Bl/6 mice), they differ in strain specific characteristics: the incubation time for mouse BSE in C57Bl/6 mice is about 177 days whereas the incubation time for mouse scrapie Me7 is 139 days in the same mouse line [25]. Moreover, BSE and scrapie display distinct differences in their glycosylation pattern [11,36] as well as the molecular mass after PK digestion (Fig. 5): The PK cleavage site of BSE-PrP^{Sc} is located further N-terminally than in Me7-PrP^{Sc}. Therefore, the residual Me7-PrP^{Sc} after PK treatment has a slightly higher molecular mass than the PK resistant part of BSE-PrP^{Sc} [35].

Both distinctive features might refer to an agent specific three dimensional structure of PrP^{Sc}, which obviously enables the pathogen to transmit its specific characteristics to the newly formed PrP^{res} during the conversion process: PrP^{res} showed a by one kDa reduced molecular mass after PK-digestion when the conversion reaction was carried out with BSE-PrP^{Sc} as a seed as compared to the banding pattern that was obtained after incubation with Me7-PrP^{Sc} as a seed. The mechanism by which such strain specific features are imparted on PrP^{res} still remains unclear. Most probably this question will not be able to be answered as long as no high-resolution structural analysis of PrP^{Sc} is available.

The observation that BSE-PrP^{Sc} but not Me7-PrP^{Sc} was capable to convert mushp-PrP^CN142S and mushp-PrP^CQ185E shows that the conversion efficiency of a PrP mutant can also strongly depend on strain specific effects. Although the precise underlying mechanism for this specific preference still remains to be elucidated, it already points at the importance of single amino acid positions for the interaction between the PrP^C template and the PrP^{Sc} seed prior to and/or during the conversion process.

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